



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C12N 15/12, 5/10, C12P 21/08</b> <b>C07K 13/00, A61K 37/02</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/09228</b> <b>(43) International Publication Date:</b> 13 May 1993 (13.05.93)
<b>(21) International Application Number:</b> PCT/US92/09326 <b>(22) International Filing Date:</b> 30 October 1992 (30.10.92)  <b>(30) Priority data:</b> 786,063 31 October 1991 (31.10.91) US  <b>(71) Applicant:</b> WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US).  <b>(72) Inventors:</b> LIN, Herbert, Y. ; 550 Memorial Drive, Apt. 12D1, Cambridge, MA 02139 (US). WANG, Xiao-Fan ; 872 Massachusetts Avenue, No 401, Cambridge, MA 02139 (US). WEINBERG, Robert, A. ; 25 Copley Street, Brookline, MA 02146 (US). LODISH, Harvey, F. ; 195 Fisher Avenue, Brookline, MA 02146 (US).		<b>(74) Agents:</b> GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).  <b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).  <b>Published</b> <i>With international search report.</i>  <b>BEST AVAILABLE COPY</b>
<b>(54) Title:</b> TGF- $\beta$ TYPE RECEPTOR cDNAS AND USES THEREFOR  <b>(57) Abstract</b>  DNA encoding TGF- $\beta$ type III receptor of mammalian origin, DNA encoding TGF- $\beta$ type II receptor of mammalian origin, TGF- $\beta$ type III receptor, TGF- $\beta$ type II receptor and uses therefor.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

## TGF- $\beta$ TYPE RECEPTOR cDNAs AND USES THEREFOR

### Description

#### Background

Transforming growth factor-beta (TGF- $\beta$ ) is a member  
5 of a family of structurally related cytokines that elicit  
a variety of responses, including growth, differentia-  
tion, and morphogenesis, in many different cell types.  
(Roberts, A.B. and M.B. Sporn, In: Peptide Growth  
Factors and Their Receptors, Springer-Verlag, Heidelberg,  
10 pp. 421-472 (1990); Massague, J., Annu. Rev. Cell. Biol.  
6:597-641 (1990)) In vertebrates at least five different  
forms of TGF- $\beta$ , termed TGF- $\beta$ 1 to TGF- $\beta$ 5, have been  
identified; they all share a high degree (60%-80%) of  
amino-acid sequence identity. While TGF- $\beta$ 1 was initially  
15 characterized by its ability to induce anchorage-  
independent growth of normal rat kidney cells, its  
effects on most cell types are anti-mitogenic. (Altschul,  
S.F. et al., J. Mol. Biol. 215:403-410 (1990); Andres,  
J.L. et al., J. Cell. Biol. 109:3137-3145 (1989)). It is  
20 strongly growth-inhibitory for many types of cells,  
including both normal and transformed epithelial, endo-  
thelial, fibroblast, neuronal, lymphoid, and hemato-  
poietic cells. In addition, TGF- $\beta$  plays a central role  
in regulating the formation of extracellular matrix and  
25 cell-matrix adhesion processes.

In spite of its widespread effects on cell phenotype  
and physiology, little is known about the biochemical  
mechanisms that enable TGF- $\beta$  family members to elicit  
these varied responses. Three distinct high-affinity

cell-surface TGF- $\beta$ -binding proteins, termed type I, II and III, have been identified by incubating cells with radiolabelled TGF- $\beta$ 1, cross-linking bound TGF- $\beta$ 1 to cell surface molecules, and analyzing the labelled complexes by polyacrylamide gel electrophoresis. (Massague, J. and  
5 B. Like, J. Biol. Chem. 260:2636-2645 (1985); Cheifetz, S. et al. J. Biol. Chem. 261:9972-9978 (1986).) The binding constants are about 5-50pM for the type I and II receptor and 30-300 pM for the type III receptor. (Boyd, F.T. and J. Massague, J. Biol. Chem. 264:2272-2278  
10 (1989))

The type I and II receptors, of estimated 53 and 70-100 kilodaltons mass respectively, are N-glycosylated transmembrane proteins that are similar in many respects. Each of these receptors has a distinct affinity for each  
15 member of the TGF- $\beta$  family of ligands. (Boyd, F.T. and J. Massague, J. Biol. Chem. 264:2272-2278 (1989)) In contrast, the type III receptor shows comparable affinities for all TGF- $\beta$  isotypes; the type III receptor is the most abundant cell-surface receptor for TGF- $\beta$  in many  
20 cell lines (upwards of 200,000 per cell), and is an integral membrane proteoglycan. It is heavily modified by glycosaminoglycan (GAG) groups, and migrates heterogeneously upon gel electrophoresis as proteins of 280 to 330 kilodaltons. When deglycosylated with heparitinase  
25 and chondroitinase, the protein core migrates as a 100-110 kilodalton protein. The TGF- $\beta$  binding site resides in this protein core, as non-glycosylated forms of this receptor that are produced in cell mutants defective in GAG synthesis are capable of ligand binding  
30 with affinities comparable to those of the natural receptor. (Cheifetz, S. and J. Massague, J. Biol. Chem., 264:12025-12028 (1989) A variant form of type III

receptor is secreted by some types of cells as a soluble molecule that apparently lacks a membrane anchor. This soluble species is found in low amounts in serum and in extracellular matrix.

The type III receptor, also called betaglycan, has a biological function distinct from that of the type I and II receptors. Some mutant mink lung epithelial cell (Mv1Lu) selected for loss of TGF- $\beta$  responsiveness no longer express type I receptors; others, similarly selected, lose expression of both the type I and II receptors. However, all these variants continue to express the type III receptor. (Boyd, F.T. and J. Massague, J. Biol. Chem. 264:2272-2278 (1989); Laiho, M. et al., J. Biol. Chem. 265:18518-18524 (1990)) This has led to the proposal that types I and II receptors are signal-transducing molecules while the type III receptor, may subserve some other function, such as in concentrating ligand before presentation to the bona fide signal-transducing receptors. The secreted form of type III receptor, on the other hand, may act as a reservoir or clearance system for bioactive TGF- $\beta$ .

Additional information about each of these TGF- $\beta$  receptor types would enhance our understanding of their roles and make it possible, if desired, to alter their functions.

## 25 Summary of the Invention

The present invention relates to isolation, sequencing and characterization of DNA encoding the TGF- $\beta$  type III receptor of mammalian origin and DNA encoding the TGF- $\beta$  type II receptor of mammalian origin. It also relates to the encoded TGF- $\beta$  type III and type II receptors, as well as to the soluble form of each; uses

of the receptor-encoding genes and of the receptors themselves; antibodies specific for TGF- $\beta$  type III receptor and antibodies specific for TGF- $\beta$  type II receptor. In particular, it relates to DNA encoding the TGF- $\beta$  type III receptor of rat and human origin, DNA encoding the TGF- $\beta$  type II receptor of human origin and homologues of each.

The TGF- $\beta$  receptor-encoding DNA of the present invention can be used to identify equivalent TGF- $\beta$  receptor type III and type II genes from other sources, using, for example, known hybridization-based methods or the polymerase chain reaction. The type III receptor gene, the type II receptor gene or their respective encoded products can be used to alter the effects of TGF- $\beta$  (e.g., by altering receptivity of cells to TGF- $\beta$  or interfering with binding of TGF- $\beta$  to its receptor), such as its effects on cell proliferation or growth, cell adhesion and cell phenotype. For example, the TGF- $\beta$  receptor type III gene, the TGF- $\beta$  receptor type II gene, or a truncated gene which encodes less than the entire receptor (e.g., soluble TGF- $\beta$  type III receptor, soluble TGF- $\beta$  type II receptor or the TGF- $\beta$  type III or type II binding site) can be administered to an individual in whom TGF- $\beta$  effects are to be altered. Alternatively, the TGF- $\beta$  type III receptor, the TGF- $\beta$  type II receptor, a soluble form thereof (i.e., a form lacking the membrane anchor) or an active binding site of the TGF- $\beta$  type III or the type II receptor can be administered to an individual to alter the effects of TGF- $\beta$ .

Because of the many roles TGF- $\beta$  has in the body, availability of the TGF- $\beta$  receptors described herein makes it possible to further assess TGF- $\beta$  function utilizing in vivo as well as in vitro methods and to alter (enhance or diminish) its effects.

### Brief Description of the Drawings

Figure 1 is the DNA sequence (SEQ ID NO. 1) and the translated amino acid sequence (SEQ ID NO. 2) of type III TGF- $\beta$ 1 receptor cDNA clone R3-OFF (full insert size 6 kb), in which the open reading frame with flanking sequences of the clone are shown. The transmembrane domain is indicated by a single underline. Peptide sequences from purified type III receptor, mentioned in text, that correspond to the derived sequence, are in italics and underlined. Potential N-linked glycosylation sites are indicated by #, and extracellular cysteines by &. A consensus protein kinase C phosphorylation site is indicated by \$. The last non-vector encoded amino acid of Clone R3-OF (2.9 kb) is indicated by @. Consensus proteoglycan attachment site is indicated by +++. Other potential glycosaminoglycan attachment sites are indicated by +. The upstream in-frame stop codon (-42 to -44) is indicated by a wavy line. Signal peptide cleavage site predicted by vonHeijne's algorithm (von Heijne, G., Nucl. Acid. Res. 14:4683-4690 (1986) is indicated by an arrow.

Figure 2 is the nucleotide sequence of the full-length type II TGF- $\beta$  receptor cDNA clone 3FF isolated from a human HepG2 cell cDNA library (full insert size 5 kb) (SEQ ID NO. 3). The cDNA has an open reading frame encoding a 572 amino acid residue protein.

Figure 3 is the amino acid sequence of the full-length type II TGF- $\beta$  receptor (SEQ ID NO. 4).

### Detailed Description of the Invention

The subject invention is based on the isolation and sequencing of DNA of vertebrate, particularly mammalian, origin which encodes TGF- $\beta$  type III receptor and DNA of mammalian origin which encodes TGF- $\beta$  type II receptor,

expression of the encoded products and characterization of the expressed products. As described, a full-length cDNA which encodes TGF- $\beta$  receptor type III has been isolated from a cDNA library constructed from a rat vascular smooth muscle cell line and a full-length cDNA which encodes TGF- $\beta$  type II receptor has been isolated from a human cDNA library. The human homologue of the type III gene has also been cloned. A deposit of human TGF- $\beta$  type III cDNA in the plasmid pBSK has been made under the terms of the Budapest Treaty at the American Type Culture Collection (10/21/91) under Accession Number 75127. All restrictions upon the availability of the deposited material will be irrevocably removed upon granting of a U.S. patent based on the subject application.

#### Isolation and Characterization of TGF- $\beta$ Type III Receptor

As described herein, two separate strategies were pursued for the isolation of the TGF- $\beta$  type III receptor cDNA. In one approach, monoclonal antibodies were generated against the type III receptor protein and used to purify the receptor, which was then subjected to microsequencing. (See Example 1) Microsequencing of several peptides resulting from partial proteolysis of the purified receptor produced four oligopeptide sequences, which were used to construct degenerate oligonucleotides. The degenerate oligonucleotides were used either as primers in a cloning strategy using the polymerase chain reaction (PCR) or as probes in screening cDNA libraries. Although this strategy did not prove to be productive, the oligopeptide sequences were useful in verifying the identity of the receptor clones isolated by the second strategy.



In the second approach to isolating TGF- $\beta$  receptor-encoding clones, an expression cloning strategy was used in COS cells; direct visualization of receptor positive cells was used to isolate receptor cDNAs. (See Example 2) In this approach, a cDNA library was constructed from  
5 A-10 cells, a rat vascular smooth muscle cell line which expresses all three TGF- $\beta$  receptors (type I, II and III). COS cells transfected with cDNA components of this library in a vector carrying the cytomegalovirus (CMV) transcriptional promoter and the SV40 origin of repli-  
10 cation were screened to identify cells expressing substantially higher than normal levels of TGF- $\beta$  receptor. One transfectant expressing such high levels of a TGF- $\beta$  binding protein was identified and the original pool of expression constructs from which it was derived was split  
15 into subpools, which were subjected to a second round of screening. Two further rounds of sib-selection resulted in isolation of one cDNA clone (R3-OF) with a 2.9 kb insert which induced high levels of TGF- $\beta$ -binding proteins in approximately 10% of cells into which it was  
20 introduced. The specificity of the TGF- $\beta$  binding was validated by showing that addition of a 200-fold excess unlabeled competitor TGF- $\beta$ 1 strongly reduced binding of  
-125 I-TGF- $\beta$  to-transfected cells.

The R3-OF cDNA encoded an open reading frame of 817  
25 amino acid residues, but did not contain a stop codon. R3-OF was used as a probe to isolate a full-length cDNA from a rat 208F library. The resulting clone, R3-OFF, is 6kb in length and encodes a protein of 853 amino acids, which is colinear with clone R3-OF. The nucleotide  
30 sequence of R3-OFF is shown in Figure 1, along with the translated amino acid sequence.

Characterization of the receptor encoded by R3-OFF was carried out, as described in Example 3. Results showed three distinct TGF- $\beta$  binding protein species of TGF- $\beta$  on the surface of mock-transfected COS cells, which is in accord with results reported by others. (Massague, J. *et al.*, Ann. NY Acad. Sci. 593:59-72 (1990)). These included the two lower molecular weight type I and II receptors (65 and 85 kD) and the higher molecular weight type III proteoglycan, which migrates as a diffuse band of 280-330 kD. Enzymatic removal of the proteoglycan yielded a core protein of approximately 100 kD. Binding to all three receptor types is specific in that it was competed by 200-fold excess of unlabeled TGF- $\beta$ 1.

Transfecting the isolated cDNA caused a two-fold increase in expression of the type III receptor. When a cell lysate derived from COS cells transfected with clone R3-OFF was treated with deglycosylating enzymes, the heterogeneous 280-330 kD band was converted to a protein core which co-migrates with the type III protein core seen in parental A10 cells. Importantly, the recombinant protein core migrated differently from the endogenous COS cell type III protein core.

These observations were confirmed and extended using stably transfected cells expressing the type III cDNA. L6 rat skeleton muscle myoblasts do not express any detectable type III mRNA and no endogenous surface type III receptor (Massague *et al.*, 1986; Segarini *et al.*, 1989). These cells were transfected with the isolated cDNA in the vector pCDNA-neo. Cell clones stably expressing this clone in both the forward and reverse orientations with respect to the CMV promoter were isolated and analyzed by ligand binding assay.

Introduction of either the full-length clone R3-OFF or the partial clone R3-OF in the forward orientation resulted in expression of type III receptor. L6 cells transfected with the cDNA clones in the reverse orientation did not express this protein. Importantly, the  
5 apparent size of the protein core of the type III receptor in cells transformed with the R3-OF clone is smaller than that from R3-OFF transformed cells, consistent with the difference in the sizes of the protein cores predicted from their nucleic acid  
10 sequences.

Surprisingly, binding of radio-labeled ligand to the type II receptor was increased by 2.5 fold in cells expressing the type III cDNA. Binding to the type I receptor was unchanged. This apparently specific  
15 up-regulation of ligand-binding to the type II receptor was evident in all of the 15 stably transfected L6 cell lines analyzed to date. Furthermore, this effect seems to be mediated equally well by the full-length clone or a truncated clone (R3-OF) that lacks the cytoplasmic domain  
20 of TGF- $\beta$  type III receptor was expressed.

Expression of type III receptor mRNA was assessed by Northern blot analysis and RNA blot analysis. Northern gel analysis showed that the type III receptor mRNA is expressed as a single 6 kb message in several rat  
25 tissues. RNA dot blot analysis of several different tissue culture cell lines was also carried out. Cells of mouse origin (MEL and YH16) appear to express a smaller (~5.5 kb) message for the type III mRNA than those of pig, rat and human origin. In all of these cells,  
30 expression or absence of the type III mRNA is consistent with the expression or absence of detectable cell surface

-10-

type III receptors, with the notable exception of the retinoblastoma cell lines (Y79, Weri-1, Weri-24, and Weri-27). These cells lack detectable surface expression of type III receptor, which confirms an earlier report. (Kimchi, A. et al., Science 240:196-198 (1988)). It is striking that the type III receptor mRNA is expressed in these cells at a level comparable to that of other cells that do indeed express type III receptor proteins at readily detectable levels. It appears that TGF- $\beta$  receptor III expression, which is substantial in normal retinoblasts (AD12), has been down-regulated in these retinoblastoma tumor cells, perhaps through post-transcriptional mechanisms.

The nucleotide sequence full reading frame along with flanking sequences of the full-length cDNA clone R3-OFF was determined and is presented in Figure 1. The reading frame encodes a protein of 853 amino acid residues, which is compatible with the 100 kD size observed for the fully deglycosylated TGF- $\beta$ 1 type III receptor. The identity of the receptor as TGF- $\beta$  type III was verified by searching for segments of the putative transcription product which included the peptide sequences determined by microsequencing of the isolated type III receptor. (See Example 1) As indicated in Figure 1, two segments of derived protein (underlined and italicized, residues 378-388 and 427-434) precisely match with the amino acid sequences of two peptides (I and III) determined from direct biochemical analysis of the purified type III receptor.

Further analysis showed that TGF- $\beta$  type III binding protein has an unusual structure for a cytokine receptor. Hydropathy analysis indicates that the protein includes a

N-terminal signal sequence, followed by a long, hydrophilic N-terminal region. A 27 residue region of strong hydrophobicity (underlined in Figure 1, residues 786-812) toward the C-terminus represents the single putative transmembrane domain. This suggests that nearly all of the receptor which is an N-terminal extracellular domain is anchored to the plasma membrane near its C-terminus. A relatively small C-terminal tail of 41 residues represents the cytoplasmic domain.

Analysis of related sequences provides few clues to function of TGF- $\beta$  type III protein. Only one other gene described to date, a glycoprotein expressed in high quantities by endothelial cells and termed endoglin, contains a related amino acid sequence. The most homologous regions between the sequences of the type III receptor and endoglin (74%) falls primarily in the putative transmembrane and cytoplasmic domains. Similar to the general structure of type III receptor, endoglin is a glycoprotein which contains a large hydrophilic N-terminal domain which is presumably extracellular, followed by a putative transmembrane domain and a short cytoplasmic tail of 47 amino acid residues. The biological role of endoglin is still unclear at present, although it has been suggested that it may involved in cell-cell recognition through interactions of an "RGD" sequence on its ectodomain with other adhesion molecules. Unlike the TGF- $\beta$  type III receptor, endoglin does not carry GAG groups.

### Isolation of TGF- $\beta$ Type II Receptor

The cDNA encoding the type II TGF- $\beta$  receptor was also isolated, using expression cloning in COS cells. A full-length cDNA (designated clone 3FF) was isolated by high stringency hybridization from a human HepG2 cell cDNA library. (See Example 6) Analysis showed that the corresponding message is a 5 kb message which is expressed in different cell lines and tissues. Sequence analysis indicated that the cDNA has an open reading frame encoding a core 572 amino acid residue protein.

10 The nucleotide sequence of the full-length type II TGF- $\beta$  receptor cDNA clone 3FF is shown in Figure 2; the amino acid sequence is represented in Figure 3.

The 572 amino acid residue protein has a single putative transmembrane domain, several consensus glycosylation sites, and a putative intracellular serine/threonine kinase domain. The predicted size of the encoded protein core is ~60 kd, which is too large for a type I TGF- $\beta$  receptor. Instead, crosslinking experiments using iodinated TGF- $\beta$  and COS cells transiently transfected with clone 3FF shows over-expression of a protein approximately 70-80 kd which corresponds to the size of type II TGF- $\beta$  receptors. Thus, clone 3FF encodes a protein that specifically binds TGF- $\beta$  and has an expressed protein size of 70-80 kd, both characteristic of

25 the type II TGF- $\beta$  receptor.

### Uses of the Cloned TGF- $\beta$ Receptors and Related Products

For the first time, as a result of the work described herein, DNAs encoding two of the three high affinity cell-surface TGF- $\beta$  receptors have been isolated, their sequences and expression patterns determined and

30

the encoded proteins characterized. Expression of the TGF- $\beta$  type III receptor in cells which do not normally express the receptor, followed by ligand binding assay, verified that the cloned type III receptor-encoding DNA (i.e., either the full-length clone R3-OFF or the partial  
5 clone R3-OF) encoded the receptor. In addition, the work described herein resulted in the surprising finding that binding of TGF- $\beta$  to type II receptors in cells expressing the type III DNA was increased by 2.5 fold.

Additional insight into the role of the TGF- $\beta$  type  
10 III receptor and its interaction with TGF- $\beta$  type II receptor is a result of the work described. For example, the role of TGF- $\beta$  type III receptor is unclear, but it has been proposed that it serves a most unusual function of attracting and concentrating TGF- $\beta$ s for eventual  
15 transfer to closely situated signal-transducing receptors. While most cytokines bind to a single cell surface receptor, members of the TGF- $\beta$  family bind with greater or lesser affinity to three distinct cell surface proteins. This has raised the question of why these  
20 three receptors are displayed by most cell types and whether they subserve distinct functions. Evidence obtained to date suggests that the type III receptor may perform functions quite different from those of types I and II. Thus, type III is substantially modified by GAGs  
25 while types I and II appear to carry primarily the N-linked (and perhaps O-linked) sidechains that are characteristic of most growth factor receptors. In addition, variant cells that have been selected for their ability to resist TGF- $\beta$ -induced growth inhibition show  
30 the absence of Type I or Type II receptors while continuing to display Type III receptors. Together, these data have caused some to propose that the Type I

and II receptors represent bona fide signal-transducing receptors while the type III receptor, described here, plays another distinct role in the cell.

It remains possible that the type III receptor serves a most unusual function of attracting and  
5 concentrating TGF- $\beta$ s on the cell surface for eventual transfer to closely situated signal-transducing receptors. Such a function would be unprecedented for a proteinaceous receptor, although heparin sulfate has been shown to activate basic FGF by binding to this growth  
10 factor prior to FGF association with its receptor (Yayon, A. et al., Cell 64:841-848 (1991)) Parenthetically, since the type III receptor also contains large quantities of heparan sulfate side-chains, it may also bind and present basic FGF to its receptor.

15 Evidence that is consistent with the role for the type III receptor comes from the work with L6 rat myoblast cells which is described herein. As described above, in L6 cells overexpressing type III receptor, the binding of radiolabelled TGF- $\beta$  to the type II receptor is  
20 increased several fold when compared with that seen with parental cells. Further assessment of TGF- $\beta$  type III function and interaction with type II and type I receptors will be needed to answer these questions and can be carried out using the materials and methods  
25 described here.

TGF- $\beta$  receptors, both type III and type II, can be identified in other species, using all or a portion of the DNA encoding the receptor to be identified as a probe and methods described herein. For example, all or a  
30 portion of the DNA sequence encoding TGF- $\beta$  type III receptor (shown in Figure 1) or all or a portion of the



DNA sequence encoding TGF- $\beta$  type II receptor (shown in Figure 2) can be used to identify equivalent sequences in other animals. Stringency conditions used can be varied, as needed, to identify equivalent sequences in other species. Once a putative TGF- $\beta$  receptor type III or type  
5 II-encoding sequence has been identified, whether it encodes the respective receptor type can be determined using known methods, such as described herein for verification that the cDNA insert of full-length clone R3-OFF and the cDNA insert of partial clone R3-OF encode  
10 the type III receptor. For example, DNA isolated in this manner can be expressed in an appropriate host cell which does not express the receptor mRNA or the surface receptor (e.g., L6 rat skeleton muscle myoblasts) and analyzed by ligand binding (TGF- $\beta$  binding) assay, as  
15 described herein.

Also as a result of the work described herein, antibodies (polyclonal or monoclonal) specific for the cloned TGF- $\beta$  type III or the clones TGF- $\beta$  type II receptor can be produced, using known methods. Such  
20 antibodies and host cells (e.g., hybridoma cells) producing the antibodies are also the subject of the present invention. Antibodies specific for the cloned TGF- $\beta$  receptor can be used to identify host cells expressing isolated DNA thought to encode a TGF- $\beta$   
25 receptor. In addition, antibodies can be used to block or inhibit TGF- $\beta$  activity. For example, antibodies specific for the cloned TGF- $\beta$  type III receptor can be used to block binding of TGF- $\beta$  to the receptor. They can be administered to an individual for whom reduction of  
30 TGF- $\beta$  binding is desirable, such as in some fibrotic diseases (e.g., of skin, kidney and lung).

-16-

The method of the present invention can be used for diagnosis of disorders involving abnormal binding of TGF- $\beta$  to TGF- $\beta$  type III receptors and/or TGF- $\beta$  type II receptors, such as fibrotic diseases. Abnormal binding of TGF- $\beta$  to TGF- $\beta$  type III receptor or TGF- $\beta$  type II receptor at a cell surface may be measured, resulting in a test binding value, which is compared to an appropriate control binding value. Control binding values can be obtained using control cells known to have abnormal binding of TGF- $\beta$  to its receptors or control cells which are normal cells (e.g., evidence TGF- $\beta$  binding to the TGF- $\beta$  receptor is within physiological levels). Control values are obtained by determining the extent to which TGF- $\beta$  binds the appropriate receptor (i.e., TGF- $\beta$  type III receptor or TGF- $\beta$  type II receptor); such values can be obtained at the time the test binding value is determined or can be previously determined (i.e., a previously determined standard). A test binding value similar to the control binding value obtained from abnormal cells is indicative of abnormal binding of TGF- $\beta$  to TGF- $\beta$  type III receptor or TGF- $\beta$  type II receptor. A test binding value similar to the control binding value obtained from normal cells is indicative of normal binding of TGF- $\beta$  to TGF- $\beta$  type III receptor or TGF- $\beta$  type II receptor.

25 DNA and RNA encoding TGF- $\beta$  type III receptor and DNA and RNA encoding TGF- $\beta$  type II receptor are now available. As used herein, the term DNA or RNA encoding the respective TGF- $\beta$  receptor includes any oligodeoxynucleotide or oligodeoxyribonucleotide sequence which, upon expression, results in production of a TGF- $\beta$  receptor having the functional characteristics of the

TGF- $\beta$  receptor. That is, the present invention includes DNA and RNA which, upon expression in an appropriate host cell, produces a TGF- $\beta$  type III receptor which has an affinity for TGF- $\beta$  similar to that of the TGF- $\beta$  type III receptor on naturally occurring cell surfaces (e.g., it shows comparable affinities for all TGF- $\beta$  isotypes). Similarly, the present invention includes DNA and RNA which, upon expression in an appropriate host cell, produces a TGF- $\beta$  type II receptor which has an affinity for TGF- $\beta$  similar to that of TGF- $\beta$  type II receptor on naturally occurring cell surfaces (e.g., it has a distinctive affinity for each member of the TGF- $\beta$  family of ligands similar to that of the naturally occurring TGF- $\beta$  type II receptor). The DNA or RNA can be produced in an appropriate host cell or can be produced synthetically (e.g., by an amplification technique such as PCR) or chemically.

The present invention also includes the isolated TGF- $\beta$  type III receptor encoded by the nucleotide sequence of full-length R3-OFF, the isolated TGF- $\beta$  type III receptor encoded by the nucleotide sequence of partial clone R3-OF, the isolated TGF- $\beta$  type II receptor encoded by the nucleotide sequence of full-length clone 3FF and TGF- $\beta$  type III and type II receptors which bind TGF- $\beta$  isotypes with substantially the same affinity. The isolated TGF- $\beta$  type III and type II receptors can be produced by recombinant techniques, as described herein, or can be isolated from sources in which they occur naturally or synthesized chemically. As used herein, the terms cloned TGF- $\beta$  type III and cloned TGF- $\beta$  type II receptors include the respective receptors identified as

-18-

described herein, and TGF- $\beta$  type III and type II receptors (e.g., from other species) which exhibit substantially the same affinity for the TGF- $\beta$  isotypes as the respective receptors.

As described previously, cells in which the cloned  
5 TGF- $\beta$  type III receptor is expressed bind TGF- $\beta$  in essentially the same manner as do cells on which the type III receptor occurs naturally. Further analysis of ligand interactions with the cloned TGF- $\beta$  type III receptor, based upon site-directed mutagenesis of both  
10 TGF- $\beta$  and the receptor, can be carried out to identify residues important for binding. For example, DNA having the sequence of Figure 1 can be altered by adding, deleting or substituting at least one nucleotide, in order to produce a modified DNA sequence which encodes a  
15 modified cloned TGF- $\beta$  type III receptor. The functional characteristics of the modified receptor (e.g., its TGF- $\beta$ -binding ability and association of the binding with effects normally resulting from binding) can be assessed, using the methods described herein. Modification of the  
20 cloned TGF- $\beta$  type III receptor can be carried out to produce, for example, a form of the TGF- $\beta$  type III receptor, referred to herein as soluble TGF- $\beta$  receptor, which is not membrane bound and retains the ability to bind the TGF- $\beta$  isotypes with an affinity substantially  
25 the same as the naturally-occurring receptor. Such a TGF- $\beta$  type III receptor could be produced, using known genetic engineering or synthetic techniques; it could include none of the transmembrane region present in the naturally-occurring TGF- $\beta$  type III receptor or only a  
30 small portion of that region (i.e., small enough not to

interfere with its soluble nature). For example, it can include amino acids 1 through 785 of the TGF- $\beta$  type III sequence of Figure 1 or a portion of that sequence sufficient to retain TGF- $\beta$  binding ability (e.g., amino acids 24-785, which does not include the signal peptide cleavage site present in the first 23 amino acids). A soluble TGF- $\beta$  type II receptor (e.g., one which does not include the transmembrane and cytoplasmic domains) can also be produced. For example, it can include amino acids 1 through 166, inclusive, of Figure 3 or a sufficient portion thereof to retain TGF- $\beta$  binding ability substantially the same as that of TGF- $\beta$  type II receptor.

The TGF- $\beta$  type III receptor and/or type II receptor can be used for therapeutic purposes. As described above, the TGF- $\beta$  family of proteins mediate a wide variety of cellular activities, including regulation of cell growth, regulation of cell differentiation and control of cell metabolism. TGF- $\beta$  may be essential to cell function and most cells synthesize TGF- $\beta$  and have TGF- $\beta$  cell surface receptors. Depending on cell type and environment, the effects of TGF- $\beta$  vary: proliferation can be stimulated or inhibited, differentiation can be induced or interrupted and cell functions can be stimulated or suppressed. TGF- $\beta$  is present from embryonic stages through adult life and, thus, can affect these key processes throughout life. The similarities of a particular TGF- $\beta$  (e.g., TGF- $\beta$ 1) across species and from cell to cell are considerable. For example, the amino acid sequence of a particular TGF- $\beta$  and the nucleotide sequence of the gene which encodes it regardless of

source, are essentially identical across species. This further suggests that TGF- $\beta$  has a critical role in essential processes.

Specifically, TGF- $\beta$  has been shown to have anti-inflammatory and immune suppression capabilities, to play an important role in bone formation (by increasing osteoblast activity), inhibit cancer cell proliferation in culture, and control proliferation of glandular cells of the prostate. As a result, it has potential therapeutic applications in altering certain immune system responses (and possibly in modifying immune-mediated diseases); in treating systemic bone disease (e.g., osteoporosis) and conditions in which bone growth is to be enhanced (e.g., repair of broken bones) and in controlling growth and metastasis of cancer cells. In addition, TGF- $\beta$  appears to play a role in determining whether some cell types undergo or do not undergo mitosis. In this respect, TGF- $\beta$  may play an important role in tissue repair. Some diseases or conditions appear to involve low production or chronic overproduction of TGF- $\beta$ . (For example, results of animal studies suggest that there is a correlation between the over production of TGF- $\beta$  and diseases characterized by fibrosis in the lung, kidney, liver or in viral mediated immune expression.)

Clearly, TGF- $\beta$  has key roles in body processes and numerous related potential clinical or therapeutic applications in wound healing, cancer, immune therapy and bone therapy. Availability of TGF- $\beta$  receptor genes, the encoded products and methods of using them in vitro and in vivo provides an additional ability to control or

regulate TGF- $\beta$  activity and effect in the body. For example, the TGF- $\beta$  type II or type III receptor encoded by the type II or the type III receptor genes of the subject invention can be used, as appropriate, to alter the effects of TGF- $\beta$  (e.g., to enhance the effect of

5 TGF- $\beta$  in the body or to inhibit or reduce (totally or partially) its effects). It is also possible to administer to an individual in whom TGF- $\beta$  bound to TGF- $\beta$  type III receptor, such as soluble TGF- $\beta$  type III receptor. The present invention provides both a TGF- $\beta$  agonist and a

10 TGF- $\beta$  antagonist. For these purposes, DNA gene encoding the entire TGF- $\beta$  type II or type III receptor, the encoded type II or type III receptor or a soluble form of either receptor can be used. Alternatively, antibodies or other ligands designed based upon these sequences or

15 specific for them can be used for this purpose.

Knowledge of the amino acid sequences of TGF- $\beta$  type III and type II receptors makes it possible to better understand their structure and to design compounds which interfere with binding of the receptor with TGF- $\beta$ . It

20 makes possible identification of existing compounds and design of new compounds which are type III and/or type II receptor antagonists.

Cells expressing the type III and/or type II receptors of the present invention can be used to screen

25 compounds for their ability to interfere with (block totally or partially) TGF binding to the receptors. For example, cells which do not express TGF- $\beta$  type III receptor (e.g., L6 rat skeleton muscle myoblasts) but have been modified to do so by incorporation of the type

30 III cDNA in an appropriate vector can be used for this

purpose. A compound to be assessed is added, for example, to tissue culture dishes containing type III expressing cells, along with labeled TGF- $\beta$ . As a control, the same concentration of labeled TGF- $\beta$  is added to tissue culture dishes containing the same type of cells. After sufficient time for binding of TGF- $\beta$  to the receptor to occur, binding of labeled TGF- $\beta$  to the cells is assessed, using known methods (e.g., by means of a gamma counter) and the extent to which it occurred in the presence and in the absence of the compound to be assessed is determined. Comparison of the two values show whether the test compound blocked TGF- $\beta$  binding to the receptor (i.e., less binding in the presence of the compound than in its absence is evidence that the test compound has blocked binding of TGF- $\beta$  to the TGF- $\beta$  type III receptor).

Alternatively, a cell line expressing the TGF- $\beta$  receptor or cells expressing microinjected TGF- $\beta$  receptor RNA, can be used to assess compounds for their ability to block TGF- $\beta$  binding to the receptor. In this embodiment, a compound to be assessed is added to tissue culture dishes containing the cell line cells expressing microinjected TGF- $\beta$  receptor RNA, along with TGF- $\beta$ . As a control, TGF- $\beta$  alone is added to the same type of cells expressing microinjected endothelin receptor RNA. After sufficient time for binding of TGF- $\beta$  to the receptor to occur, the extent to which binding occurred is measured, both in the presence and in the absence of the compound to be assessed. Comparison of the two values shows whether the compound blocked TGF- $\beta$  binding to the receptor. The TGF- $\beta$  type III and type II receptors can



also be used to identify TGF- $\beta$ -like substances, to purify  
TGF- $\beta$  and to identify TGF- $\beta$  regions which are responsible  
for binding to the respective receptors. For example,  
the type III receptor can be used in an affinity-based  
5 method to identify substances which bind the receptor in  
a manner similar to TGF- $\beta$ .

The invention will now be illustrated by the  
following examples, which are not intended to be limiting  
in any way.

10

#### EXAMPLES

Materials and methods used in Examples 1-5 are  
described below.

#### Materials

15 The following is a description of materials used in  
the work described herein.

Recombinant human TGF- $\beta$ 1 was provided by Rik Derynck  
of Genentech. COS-M6 cells were provided by Brian Seed  
of the Massachusetts General Hospital and Alejandro  
Aruffo of Bristol-Myers-Squibb. Heparitinase was pro-  
20 vided by Tetsuhito Kojima and Robert Rosenberg of MIT.  
LLC-PK<sub>1</sub> cells were a gift of Dennis Ausiello of the  
Massachusetts General Hospital. YH-16 cell were a gift  
of Edward Yeh of the Massachusetts General Hospital. 3-4  
cells were a gift of Eugene Kaji of the Whitehead  
25 Institute for Biomedical Research. All other cell lines  
were purchased from ATCC and grown as specified by the  
supplier, except as noted.

## Expression Cloning

### Construction of cDNA Library and Generation of Plasmid Pools

10 $\mu$ g polyadenylated mRNA was prepared from A10 cells  
5 by the proteinase-K/SDS method (Gonda et al., Molec.  
Cell. Biol. 2:617-624 (1982)). Double stranded cDNA was  
synthesized and linkered to nonpalindromic BstX1 adaptors  
as described by Seed, B. and A. Aruffo, Proc. Natl. Acad.  
Sci. USA 84:3365-3369 (1987). Acaptured cDNA was size-  
10 fractionated on a 5 to 20% potassium acetate gradient,  
and inserts greater than 1 kb were ligated to the plasmid  
vector pcDNA-1, and electroporated in the E. coli  
MC1061/P3, yielding a primary library with a titer of  
>10<sup>7</sup> recombinants. A portion of the cDNA was plated as  
15 pools of  $\sim 1 \times 10^4$  recombinant bacteria colonies grown on 15  
cm petri dishes with Luria-broth agar containing 7.5  
mg/ml tetracycline and 12.5 mg/ml ampicillin. Cells were  
scraped off the plates in 10 mls of Luria-broth, and  
glycerol stocks of pooled bacteria were stored at -70°C.  
20 The remaining bacteria was used to purify plasmid DNA  
using the alkaline lysis mini-prep method (Sambrook, J.  
et al., Molecular Cloning: A Laboratory Manual, 2d Ed.  
(Cold Spring Harbor, NY, Cold Spring Harbor Laboratory  
Press (1989)).

### 25 COS Cell Transfections and Binding Assay

Plasmid pools (each representing  $\sim 1 \times 10^4$  clones) were  
transfected into COS-M6 (subclone of COS-7 cells) by the  
DEAE-dextran/chloroquine method described by Seed, B. and  
A. Aruffo, Proc. Natl. Acad. Sci. USA 84:3365-3369  
30 (1987). Forty-eight hours after transfection, cells were

incubated with 50 pM<sup>125</sup>I-TGF- $\beta$ 1 (100 to 200 Ci/mmol) for  
4 hours at 4°C), autoradiographic analysis of transfected  
cells was performed using NT-B2 photographic emulsion  
(Kodak) essentially as described (Gearing, D.P. et al.,  
5 EMBO J. 8:3667-3676 (1989)). After development of  
slides, cells were air-dried and mounted with mounting  
media and a glass coverslip. Slides were analyzed under  
an Olympus OM-T1 inverted phase-contrast microscope using  
a dissection trans-illuminator for darkfield illumi-  
10 nation.

#### Subdivision of Positive Pool

Of 86 pools screened, one pool (#13) was identified  
as positive and a glycerol stock of bacteria corres-  
ponding to this pool was titered and 25 pools of 1000  
15 clones were generated and miniprep plasmid from these  
pools were transfected into COS cells as described above.  
Several positive pools of 1000 were identified, and one  
was replated as 25 plates of 100 colonies. A replica was  
made of this positive plate and harvested. Once a  
20 positive pool was identified, individual colonies were  
picked from the corresponding master plate and grown  
overnight in 3 ml liquid culture. A 2-dimensional grid  
representing the 100 clones was generated and a single  
clone, R3-OF, was isolated.

#### 25 Cloning of R3-OFF

A 208F rat fibroblast library in lambda ZAP II  
(Stratagene) was screened at high stringency with clone  
R3-OF insert, and several clones with ~6kb inserts were  
isolated, one of which is referred to as R3-OFF.

### DNA Sequencing and Sequence Analysis

Double-stranded DNA was sequenced by the dideoxy. chain termination method using Sequenase reagents (United States Biochemicals). Comparison of the sequence to the data bases was performed using BLAST (Altschul, S.F. et al., J. Mol. Biol. 215:403-410 (1990)).

### Iodination of TGF- $\beta$

TGF- $\beta$ 1 was iodinated using the chloramine-T method as described (Cheifetz, S. and J.L. Andres, J. Biol. Chem. 263:16984-16991 (1988)).

### Chemical Cross-Linking

Transfected COS cells grown on 10 cm dishes or subconfluent L6 and A-10 cells grown on 3.5 cm dishes were incubated with  $^{125}\text{I}$ -TGF- $\beta$ 1 in binding buffer (Frebs-Ringer buffered with 20 mM Hepes, pH 7.5, 5 mM  $\text{MgSO}_4$ , 0.5% BSA), washed 4 times with ice-cold binding buffer without BSA, and incubated for 15 minutes with binding buffer without BSA containing 60ng/ml disuccinimidyl suberate at 4°C under constant rotation. Crosslinking was terminated by addition of 7% sucrose in binding buffer. Cells were scraped, collected and pelleted by centrifugation, then resuspended in lysis buffer (10 mM Tris, pH 7.4, 1 mM EDTA, pH 8.0, 1% Triton-X 100, 10  $\mu\text{g/ml}$  of pepstatin, 10 $\mu\text{g/ml}$  leupeptin, 10  $\mu\text{g/ml}$  antipain, 100  $\mu\text{g/ml}$ ; benzamidine hydrochloride, 100  $\mu\text{g/ml}$  soybean trypsin inhibitor, 50  $\mu\text{g/ml}$  aprotonin, and 1 mM phenylmethanesulfonyl fluoride). Solubilized material was analyzed by 7% SDS-PAGE and subjected to

autoradiographic analysis by exposure to X-AR film  
(Kodak) at -70°C.

#### Enzymatic Digestion

Digestion of solubilized TGF- $\beta$  receptors with  
5 chondroitinase and heparitinase was performed as  
described (Cheifetz, S. and J.L. Andres, J. Biol. Chem.  
263:16984-16991 (1988); Segarini, P.R. and S.M. Seyedin,  
J. Biol. Chem., 263: 8366-8370 (1988).

#### Generation of Stable Cell Lines

10 L6 myoblasts were split 1:10 into 10 cm dishes and  
transfected the following day by the calcium phosphate  
method (Chen, C. and H. Okayama, Molec. Cell. Biol.  
7:2745-2752 (1987)) with clones R3-OF or R3-OFF in the  
forward and reverse orientations in the vector pcDNA-neo  
15 (InVitrogen). Cells were subjected to selection in the  
presence of G418 (Geneticin, GIBCO) for several weeks  
until individual colonies were visible by the naked eye.  
These clones were isolated and amplified.

#### RNA Blot Analyses

20 Rat tissue polyadenylated mRNA was prepared by the  
lithium chloride/urea method (Auffrey, C. and F. Raugeon,  
Eur. J. Biochemistry 107:303-313 (1980), followed by  
oligo-dT cellulose chromatography (Aviv and Leder, 1972).  
Polyadenylated mRNA from cell lines was prepared by the  
25 proteinase K/SDS method (Gonda, T.J. et al., Molec. Cell.  
Biol. 2:617-624 (1982)). Samples of mRNA were resolved  
by electrophoresis on 1% agarose-2.2M formaldehyde gels,  
blotted onto nylon membranes (Biotrans, ICN) and incubated

-28-

with the 2.9 kb insert of clone Re-OF labeled with  $^{32}\text{P}$  by random priming as probe (Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2d Ed., Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, (1989).  
5 Hybridizations were performed at  $42^{\circ}\text{C}$  in hybridization buffer containing 50% formamide overnight, and blots were washed at  $55^{\circ}\text{C}$  in 0.2X SSC, 0.1% SDS, before exposure to X-AR film at  $-70^{\circ}\text{C}$ .

10 Example 1. Production of Anti-Type III Receptor Protein Antibodies and Microsequencing and Microsequencing of Peptides Resulting from Partial Proteolysis of Purified Type III Receptor

Initially cellular proteoglycans were purified from human placenta and then subjected to enzymatic deglycosylation with heparitinase and chondroitinase. Protein  
15 cores in the molecular weight range of 100-130 kilodaltons were further purified by preparative gel electrophoresis; these should include the type III receptor. This partially purified material was used as an immunogen  
20 in mice. After screening 850 hybridoma lines developed from immunized mice, three lines were found to produce antibodies that specifically recognized and immunoprecipitated a deglycosylated polypeptide species of 100-120 kD. This species could be radiolabelled by  
25 incubation of whole cells with  $^{125}\text{I}$ -TGF- $\beta$  followed by covalent cross-linking. Its size is consistent with that of the protein core previously reported for the type III receptor. (Massague, J., Annu. Rev. Cell. Biol. 6:597-641 (1990))

Monoclonal antibody 94 was used to purify the type  
-III receptor from rat liver by affinity-chromatography.  
The purified receptor was subjected to partial proteo-  
lysis and the resulting peptides were resolved by high  
5 pressure liquid chromatography. Several peptides were  
subjected to microsequencing and yielded the following  
oligopeptide sequences:

Peptide I: ILLDPDHPPAL (SEQ ID NO. 5)

Peptide II: QAPFPINFMIA (SEQ ID NO. 6)

10 Peptide III: QPIVPSVQ (SEQ ID NO. 7)

Peptide IV: FYVEQGYGR (SEQ ID NO. 8)

These peptide sequences were used to construct  
degenerate oligonucleotides that served either as primers  
in a cloning strategy using the polymerase chain reaction  
15 (PCR) or as probes in screening cDNA libraries. While  
this strategy was not productive, the oligopeptide  
sequences proved useful in verifying the receptor clones  
isolated by the second, alternative strategy (see Example  
2).

## 20 Example 2. Expression Cloning of the Type III Receptor cDNA

An expression cloning strategy in COS cells, a  
procedure which takes advantage of the considerable  
amplification of individual cDNAs in transfected COS  
25 cells was used as an alternative means to isolate TGF- $\beta$   
receptor clones. Such amplification is mediated by SV40  
large T antigen expressed by the COS cells interacting

with a SV40 origin of replication in the cDNA vector. Gearing, D. et al., EMBO J. 8:3667-3676 (1989); Lin, H.Y., et al., Proc. Natl. Acad. Sci. 88:3185-3189 (1991a); Lin, H. Y. et al., Science, in press (1991); Mathews, L. S. and Vale, W. W., Cell 65:973-982 (1991).

5        The strategy involved the construction of a cDNA library from A-10 cells, a rat vascular smooth muscle cell line that expresses all three high-affinity TGF- $\beta$  receptors. The resulting cDNAs were inserted into the vector pCDNA-1, which carries the CMV transcriptional  
10 promoter and the SV40 origin of replication. The resulting library was then divided into pools of 10,000 independent recombinants each and DNA from each pool was transfected into  $1.5 \times 10^6$  COS-7 cells grown on glass flaskettes by means of DEAE-dextran transfection procedure. Aruffo, A. and Seed, B., Proc. Natl. Acad. Sci., U.S.A. 84:8573-8577 (1987); Gearing, D. et al., EMBO J. 8:3667-3676 (1989); Mathews, L. S. and Vale, W. W., Cell 65:973-982 (1991). The transfected cells were cultured  
15 for 48-60 hours and then exposed to radiolabelled TGF- $\beta$ 1 for four hours. Following this treatment, the glass slides carrying these cells were washed extensively and fixed. These slides were dipped in liquid photographic emulsion and examined by darkfield microscopy. While all  
20 of the receptor genes cloned to date by this procedure have undetectable or low constitutive levels of expression in COS cells, we were hindered by the fact that untransfected COS cells already express substantial amounts of type III TGF- $\beta$  receptor. Such expression, estimated to be approximately  $2 \times 10^5$  receptor molecules  
25 per cell, might well have generated an unacceptably high level of background binding. However, since the detection procedure involves visualizing radiolabelled  
30



ligand-binding on individual cells, it was hoped that identifying occasional cells expressing substantially higher levels of vector-encoded receptor would be possible. This hope was vindicated in the initial experiments.

5       After screening nearly one million cDNA clones in this manner, a glass slide containing 20 positive transfectants was identified. The original pool of expression constructs from which one such transfectant was derived was split into 25 subpools of 1000 clones each and these  
10       were subjected to a second round of screening. Two further rounds of sib-selection resulted in the isolation of a cDNA clone (R3-OF) with a 2.9 kb insert that induced high levels of TGF- $\beta$ -binding proteins in approximately 10% of COS cells into which it was transfected.

15       The specificity of this binding was validated by showing that addition of a 200-fold excess of unlabeled TGF- $\beta$  competitor strongly reduced binding of  $^{125}$ I-TGF- $\beta$  to transfected cells. By taking into account a transfection efficiency of 10% and the high background of  
20       endogenous receptor expression, we calculated that the level of total  $^{125}$ I-TGF- $\beta$  binding to each glass slide of cells transfected with this cDNA clone (Figure 1C) was only 2-fold above the level seen with mock transfectants (data not shown). Nonetheless, this marginal increase in  
25       ligand-binding was sufficient to identify rare transfectants amidst a large field of cells expressing this background level of receptor.

      The R3-OF cDNA encoded an open reading frame of 836 amino acid residues of which the 3' most 18 were encoded  
30       by vector sequence, clearly indicating that clone R3-OF

was an incomplete cDNA insert which ended prematurely at the codon specifying alanine 818 (Figure 4). R3-OF was used as a probe to isolate a full-length cDNA from a rat 208F lambda phage library. This clone, termed R3-OFF, was 6 kb in length and encoded a protein of 853 amino acids; its sequence was co-linear with that of clone R3-OF.

Example 3. Characterization of the Product of the Full Length Clone R3-OFF

Characterization of the product of the full length clone R3-OFF was undertaken in order to determine which of the three TGF- $\beta$  receptors it specified. To do so, COS transfectants were incubated with radioiodinated TGF- $\beta$ , chemical crosslinker was added and the labelled receptors were resolved by polyacrylamide gel electrophoresis.

Labelling of cell surface TGF- $\beta$  receptors in this way resulted in the detection of three distinct species on the surface of COS cells, as extensively by others (Massague, J. et al., Ann. NY Acad. Sci. 593:59-72 (1990)). These included the two lower molecular weight type I and II receptors (65 and 85 kD) and the higher molecular weight type III proteoglycan, which migrated as a diffuse band of 280-330 kD. Enzymatic treatment of the proteoglycan with chondroitinase and heparitinase yielded a core protein of approximately 100 kD. Binding to all three receptor types was specific, in that it was completed by 200-fold excess of unlabeled TGF- $\beta$ 1.

Transfecting the R3-OFF cDNA caused a two-fold increase in expression of the type III receptor. When a cell lysate derived from COS cells transfected with clone

R3-OFF was treated with deglycosylating enzymes, the heterogenous 280-330 kd band was converted to a protein core which co-migrated with the type III protein core seen in untransfected A10 cells. Importantly, the recombinant protein core migrates differently from the  
5 endogenous COS cell type III protein core.

These observations were confirmed and extended in experiments using stably transfected cells expressing the R3-OFF cDNA. L6 rat skeleton muscle myoblasts normally do not express detectable type III mRNA or endogenous  
10 type III receptor (determined by radiolabelled ligand-binding assay). Such cells were transfected with the isolated cDNA in the vector pCDNA-neo. Cell clones stably expressing this clone in both the forward and reverse orientations with respect to the CMV promoter  
15 were isolated and analyzed by ligand-binding assay.

Introduction of either the full length clone R3-OFF or the partial clone R3-OF in the forward orientation led to the de novo expression of the type III receptor. L6 cells transfected with the cDNA in reversed orientation  
20 did not express this protein. The apparent size of the protein core of the type III receptor in cells transfected with the R3-OF clone is smaller than that expressed by R3-OFF transfected cells, consistent with the difference in the sizes of the protein cores  
25 predicted from the respective nucleic acid sequences (Figure 1).

Unexpectedly, the amount of radio-labelled ligand cross-linked to the type II receptor is increased by 2.5 fold in cells expressing the type III cDNA, while the  
30 amount cross-linked to the type I receptor remained

unchanged. This apparent specific up-regulation of ligand-binding to the type II receptor could be detected with all of the 15 stably transfected L6 cell lines analyzed so far. This effect seems to be mediated by the truncated clone R3-OF which lacks the cytoplasmic domain as well as by the full-length clone R3-OFF.

Example 4. Expression of Type III Receptor

Northern blot analysis demonstrated that the type III receptor mRNA is expressed as a single 6 kb message in several rat tissues. The level of mRNA expression in the liver was less than in other tissues, a result expected from earlier surveys of various tissues using radioiodinated TGF- $\beta$ 1. Based on this information, it appears that clone R3-OFF, with a ~6 kb cDNA insert, represents a full length rat type III cDNA clone.

Cells of mouse origin (MEL and YH16) appear to express a smaller (~5.5 kb) message for the type III mRNA than those of pig, rat and human origin. In all of these cells, expression or absence of the type III mRNA is consistent with the expression or absence of detectable cell surface type III receptors with the notable exception of the retinoblastoma cell lines (Y79, Weri-1, Weri-24, and Weri-27). These cells have previously been shown to lack detectable surface expression of type III receptor, a result confirmed by our own unpublished work. It is striking that the type III receptor mRNA is expressed in these cells at a level comparable to that of other cells that do indeed express type III receptor proteins at readily detectable levels. At this moment, we can only conclude that TGF- $\beta$  receptor III expression,

which is substantial in normal retinoblasts (AD12), has been down-regulated in these retinoblastoma tumor cells, perhaps through post-transcriptional mechanisms.

Example 5. Sequence Analysis of the Full-Length Type  
III cDNA

5

The full-length cDNA clone (R3-OFF), described in Example 4, was subjected to sequence analysis. The full reading frame along with flanking sequences is presented in Figure 1. This reading frame encodes a protein of 853  
10 amino acid residues, which is compatible with the 100 kD observed for the fully deglycosylated TGF- $\beta$  type III receptor.

Two segments of derived protein sequence (underlined and italicized, residues 378-388 and 427-434) precisely  
15 match those determined earlier from direct biochemical analysis of the purified receptor protein. This further secured the identity of this isolated cDNA clone as encoding the rat type III receptor.

This TGF- $\beta$  binding protein has an unusual structure  
20 for a cytokine receptor. Hydropathy analysis indicates a N-terminal signal sequence, followed by a long, hydrophilic N-terminal region (Kyte, J. and R. F. Doolittle, J. Mol. Biol. 157:105-132 (1982)). A 27  
25 residue region of strong hydrophobicity (underlined, residues 786-812) toward the C-terminus represents the single putative transmembrane domain. This suggests that nearly all of the receptor is composed of an N-terminal extracellular domain that is anchored to the plasma membrane near its C-terminus. A relatively short

C-terminal tail of 41 residues represents the putative cytoplasmic domain.

The clone R3-OF was also analyzed and found to be a truncated version of R3-OFF, with an identical open reading frame but whose last encoded residue is alanine  
5 818 (Figure 1).

In R3-OFF there are six consensus N-linked glycosylation sites and 15 cysteines (indicated in Figure 1). There is at least one consensus glycosaminoglycan addition site at serine 535 (Bernfield, M. and K. C.  
10 Hooper, Ann. N.Y. Acad. Sci. in press (1991), and numerous Ser-Gly residues that are potential sites for GAG conjugation. A consensus protein kinase C site is also present at residue 817.

Only one other gene described to date, a  
15 glycoprotein expressed in high quantities by endothelial cells and termed endoglin (Gougos and Letarte, 1990), contains a related amino acid sequence. Overall, there is -30% identity with the type III receptor over the entire 645 amino acid residue length of endoglin. The  
20 most homologous regions between the sequences of the type III receptor and endoglin (74% identity) falls primarily in the putative transmembrane and cytoplasmic domains. Similar to the general structure of type III receptor, endoglin is a glycoprotein which contains a large  
25 hydrophilic and presumably extracellular N-terminal domain followed by a putative transmembrane domain and a short cytoplasmic tail of 47 amino acid residues. The biological role of endoglin is unclear, though it has been suggested that it may involve cell-cell recognition  
30 through interactions of an "RGD" sequence on its

ectodomain with other adhesion molecules. Unlike the ~~TGF- $\beta$ -type-III~~ receptor, endoglin does not carry GAG groups.

Equivalents

- 5        Those skilled in the art will recognize, or be able to ascertain using not more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

International Application No: PCT/

<b>MICROORGANISMS</b>	
Optional Sheet in connection with the microorganism referred to on page <u>6</u> , line <u>12</u> of the description *	
<b>A. IDENTIFICATION OF DEPOSIT *</b> Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution * <b>American Type Culture Collection</b>	
Address of depositary institution (including postal code and country) * <b>12301 Parklawn Drive            Rockville, Maryland 20852 USA</b>	
Date of deposit * <b>21 October 1991</b>	Accession Number * <b>75127</b>
<b>B. ADDITIONAL INDICATIONS *</b> (leave blank if not applicable). This information is continued on a separate attached sheet <input type="checkbox"/>	
<p>In respect of those designations in which a European Patent is sought, the Applicant hereby informs the European Patent Office under European Rule 28(4) that, until the publication of the mention of the grant of the European Patent or until the date on which the European Application has been refused or is withdrawn or is deemed to be withdrawn, the availability of the biological material deposited with the American Type Culture Collection under Accession No. shall be effected only by the issue of a sample to an expert nominated by the requester in accordance with European Rule 28(5).</p>	
<b>C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *</b> (if the indications are not for all designated States)	
Europe (EP) Australia Canada Japan	
<b>D. SEPARATE FURNISHING OF INDICATIONS *</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g. "Accession Number of Deposit")	
<b>E.</b> <input checked="" type="checkbox"/> This sheet was received with the international application when filed (to be checked by the receiving Office)	
<b>FRANZ L. BÖNNER</b> <b>INTERNATIONAL DIVISION</b> (Authorized Officer)	
<input type="checkbox"/> The date of receipt (from the applicant) by the International Bureau is	
<div style="display: flex; justify-content: space-between;"> <span>was</span> <span>_____ (Authorized Officer)</span> </div>	



CLAIMS

1. Isolated DNA encoding TGF- $\beta$  receptor of vertebrate origin or DNA which hybridizes thereto and encodes TGF- $\beta$  receptor of vertebrate origin.
- 5 2. Isolated DNA of Claim 1 wherein the TGF- $\beta$  receptor is TGF- $\beta$  type III receptor or TGF- $\beta$  type II receptor.
3. Isolated DNA of Claim 2 which is of mammalian origin.
- 10 4. Isolated DNA of murine or human origin encoding TGF- $\beta$  type III receptor or DNA which hybridizes thereto.
- 15 5. Isolated DNA of Claim 4 having the nucleotide sequence of Figure 1 or a portion thereof sufficient to encode TGF- $\beta$  type III receptor.
6. Isolated DNA of murine or human origin encoding TGF- $\beta$  type II receptor or DNA which hybridizes thereto.
- 20 7. Isolated DNA of Claim 6 having the nucleotide sequence of Figure 2 or a portion thereof sufficient to encode TGF- $\beta$  type II receptor.
8. Isolated TGF- $\beta$  type III receptor of mammalian origin.

9. Isolated TGF- $\beta$  type III receptor of Claim 8 having the amino acid sequence of Figure 1 or a substantially similar amino acid sequence.
10. Isolated TGF- $\beta$  type II receptor of mammalian origin.
- 5 11. Isolated TGF- $\beta$  type II receptor of Claim 10 having the amino acid sequence of Figure 3 or a substantially similar amino acid sequence.
12. Recombinant TGF- $\beta$  type III receptor of mammalian origin.
- 10 13. Recombinant TGF- $\beta$  type III receptor of Claim 8 having the amino acid sequence of Figure 1 or a substantially similar amino acid sequence.
14. Recombinant TGF- $\beta$  type II receptor of mammalian origin.
- 15 15. Recombinant TGF- $\beta$  type II receptor of Claim 10 having the amino acid sequence of Figure 4 or a substantially similar amino acid sequence.
16. Soluble TGF- $\beta$  receptor.
17. Soluble TGF- $\beta$  receptor of Claim 16 which is soluble  
20 TGF- $\beta$  type III receptor.
18. Soluble TGF- $\beta$  type III receptor of Claim 17 in which the amino acid sequence is amino acids 1 through

785, inclusive, of Figure 1 or a substantially similar amino acid sequence.

19. Soluble TGF- $\beta$  receptor of Claim 16 which is soluble TGF- $\beta$  type II receptor.
- 5 20. Soluble TGF- $\beta$  receptor of Claim 19 in which the amino acid sequence is approximately amino acids 1 through 166, inclusive, of Figure 3, or a substantially similar amino acid sequence.
- 10 21. An antibody which specifically recognized TGF- $\beta$  type III receptor of mammalian origin.
22. An antibody of Claim 21 which is a monoclonal antibody.
23. An antibody which specifically recognizes soluble TGF- $\beta$  type III receptor of mammalian origin.
- 15 24. An antibody which specifically recognizes soluble TGF- $\beta$  type II receptor of mammalian origin.
- 20 25. A method of altering TGF- $\beta$  binding to TGF- $\beta$  type II or type III receptor on the surface of a cell, comprising combining soluble TGF- $\beta$  type II or type III receptor with the cell, under conditions appropriate for binding of the soluble TGF- $\beta$  receptor and TGF- $\beta$ .

-42-

26. The method of Claim 25 wherein TGF- $\beta$  binding is inhibited.
27. A method of altering TGF- $\beta$  binding to TGF- $\beta$  type III receptor on the surface of a cell comprising  
5 combining the cell with DNA encoding TGF- $\beta$  type III receptor in an appropriate expression system which expresses TGF- $\beta$  type III receptor, under conditions appropriate for expression of TGF- $\beta$  type III receptor and binding of TGF- $\beta$  with TGF- $\beta$  type III  
10 receptor.
28. A method of regulating the effect of TGF- $\beta$  in a mammal, comprising administering to the mammal a TGF- $\beta$  receptor selected from the group consisting  
15 of: TGF- $\beta$  type III receptor, TGF- $\beta$  type II receptor, soluble TGF- $\beta$  type III receptor, soluble TGF- $\beta$  type II receptor, TGF- $\beta$  bound to TGF- $\beta$  type III receptor or a combination thereof, in sufficient quantity to alter binding of TGF- $\beta$  to TGF- $\beta$  type III  
20 receptor, binding of TGF- $\beta$  to TGF- $\beta$  type II receptor or both.

29. TGF- $\beta$  receptor according to any one of Claims 8 to 20, for use in therapy.
30. An antibody according to any one of Claims 21 to 24, for use in therapy.
- 5 31. Use of TGF- $\beta$  receptor according to any one of Claims 8 to 20, for the manufacture of a medicament for altering (e.g. inhibiting) TGF- $\beta$  binding to TGF- $\beta$  type II or type III receptor on the surface of a cell.
- 10 32. Use of a TGF- $\beta$  receptor selected from the group consisting of: TGF- $\beta$  type III receptor, TGF- $\beta$  type II receptor, soluble TGF- $\beta$  type III receptor, soluble TGF- $\beta$  type II receptor, TGF- $\beta$  bound to TGF- $\beta$  type III receptor or a combination thereof, for the  
15 manufacturing of a medicament for use in regulating the affect of TGF- $\beta$  in a mammal.
33. A method of assessing the ability of a compound to interfere with TGF- $\beta$  binding to the TGF- $\beta$  type III receptor, comprising the steps of:  
20 a) combining:  
1) mammalian cells which express the TGF- $\beta$  type III receptor;  
2) labeled TGF- $\beta$ ; and  
3) a compound to be assessed;

- 5           b) maintaining the product of (a) under conditions  
            sufficient for TGF- $\beta$  to bind to the TGF- $\beta$  type  
            III receptor;
- c) determining the extent of binding of TGF- $\beta$  to  
            TGF- $\beta$  type III receptors in the presence of the  
            compound to be assessed; and
- 10           d) comparing the determination made in (c) with  
            the extent to which binding of TGF- $\beta$  to the  
            TGF- $\beta$  type III receptor occurs in the absence  
            of the compound to be assessed,
- wherein if TGF- $\beta$  binding to the TGF- $\beta$  type III  
            receptor occurs to a lesser extent in the presence  
            of the compound to be assessed than in the absence  
15           of the compound to be assessed, the compound to be  
            assessed interferes with TGF- $\beta$  binding to TGF- $\beta$  type  
            III receptors.
34. A method of Claim 33 wherein the cells which express  
the TGF- $\beta$  type III receptor are a cell line.
- 20           35. A method of Claim 34 wherein the cells which express  
            the TGF- $\beta$  type III receptor are cells modified to  
            express the TGF- $\beta$  type III receptor.
- 25           36. A method of Claim 35 wherein the cells modified to  
            express the TGF- $\beta$  type III receptor are cells which  
            have incorporated into them TGF- $\beta$  receptor cDNA in  
            an appropriate vector or microinjected TGF- $\beta$   
            receptor RNA.

37. A method of assessing the ability of a compound to interfere with TGF- $\beta$  binding to the TGF- $\beta$  type II receptor comprising the steps of:

a) combining:

5

- 1) mammalian cells which express the TGF- $\beta$  type II receptor;
- 2) labeled TGF- $\beta$ ; and
- 3) a compound to be assessed;

b) maintaining the product of (a) under conditions sufficient for TGF- $\beta$  to bind to the TGF- $\beta$  type II receptor;

10

c) determining the extent of binding of TGF- $\beta$  to TGF- $\beta$  type II receptors in the presence of the compound to be assessed; and

15

d) comparing the determination made in (c) with the extent to which binding of TGF- $\beta$  to the TGF- $\beta$  type II receptor occurs in the absence of the compound to be assessed,

20

wherein if TGF- $\beta$  binding to the TGF- $\beta$  type II receptor occurs to a lesser extent in the presence of the compound to be assessed than in the absence of the compound to be assessed, the compound to be assessed has interfered with TGF- $\beta$  binding to TGF- $\beta$  type II receptor.

25

38. A method of Claim 37 wherein the cells which express the TGF- $\beta$  type II receptor are a cell line.

39. A method of Claim 38 wherein the cells which express the TGF- $\beta$  type II receptor are cells modified to express the TGF- $\beta$  type II receptor.

40. A method of Claim 39 wherein the cells modified to express the TGF- $\beta$  type II receptor are cells which have incorporated into them TGF- $\beta$  receptor cDNA in an appropriate vector or microinjected TGF- $\beta$  receptor RNA.
- 5
41. A method of detecting abnormal binding of TGF- $\beta$  TGF- $\beta$  type III receptors of TGF- $\beta$  type II receptors at a cell surface, comprising:
- 10 a) determining the extent of binding of TGF- $\beta$  to TGF- $\beta$  type III receptors or TGF- $\beta$  type II receptors by cells in a sample obtained from an individual in whom binding is to be assessed thereby producing a test binding value; and
- 15 b) comparing the results of (a) with the extent to which binding occurs at the cell surface in control cells which are cells known to have abnormal binding of TGF- $\beta$  to TGF- $\beta$  type III receptors or TGF- $\beta$  type II receptors resulting in a control binding value,
- 20 wherein a test binding value similar to the control binding value is indicative of abnormal binding of TGF- $\beta$  to TGF- $\beta$  type III receptor or TGF- $\beta$  type II receptor.



FIGURE 1A

-240

CAGGAGGTGA AAGTCCCCGG CGGTCCGGAT GCGGCAGTTG CACTGCGCTG CTGAGCTCGC -180  
 GGCCGCCTGC GCACACTGGG GGGACTCGCT TCGGCTAGTA ACTCCTCCAC CTCGCGGCGG -120  
 ACGACCGGTC CTGGACACGC TGCCTGCGAG GCAAGTTGAA CAGTGCAGAG AAGGATCTTA - 60  
 AAGCTACACC CGACTTGCCA CGATTGCCTT CAATCTGAAG AACCAAAGGC TGTTGGAGAG - 1

---

ATG GCA GTG ACA TCC CAC CAC ATG ATC CCG GTG ATG GTT GTC CTG ATG	48
Met Ala Val Thr Ser His His Met Ile Pro Val Met Val Val Leu Met	16
AGC GCC TGC CTG GCC ACC GCC GGT CCA GAG CCC AGC ACC CGG TGT GAA	96
Ser Ala Cys Leu Ala Thr Ala Gly Pro Glu Pro Ser Thr Arg Cys Glu	32
	&
CTG TCA CCA ATC AAC GCC TCT CAC CCA GTC CAG GCC TTG ATG GAG AGC	144
Leu Ser Pro Ile Asn Ala Ser His Pro Val Gln Ala Leu Met Glu Ser	48
	#
TTC ACC GTT CTG TCT GGC TGT GCC AGC AGA GGC ACC ACC GGG CTG CCA	192
Phe Thr Val Leu Ser Gly Cys Ala Ser Arg Gly Thr Thr Gly Leu Pro	64
	+ &
AGG GAG GTC CAT GTC CTA AAC CTC CGA AGT ACA GAT CAG GGA CCA GGC	240
Arg Glu Val His Val Leu Asn Leu Arg Ser Thr Asp Gln Gly Pro Gly	80
CAG CGG CAG AGA GAG GTT ACC CTG CAC CTG AAC CCC ATT GCC TCG GTG	288
Gln Arg Gln Arg Glu Val Thr Leu His Leu Asn Pro Ile Ala Ser Val	96
CAC ACT CAC CAC AAA CCT ATC GTG TTC CTG CTC AAC TCC CCC CAG CCC	336
His Thr His His Lys Pro Ile Val Phe Leu Leu Asn Ser Pro Gln Pro	112
CTG GTG TGG CAT CTG AAG ACG GAG AGA CTG GCC GCT GGT GTC CCC AGA	384
Leu Val Trp His Leu Lys Thr Glu Arg Leu Ala Ala Gly Val Pro Arg	128
CTC TTC CTG GTT TCG GAG GGT TCT GTG GTC CAG TTT CCA TCA GGA AAC	432
Leu Phe Leu Val Ser Glu Gly Ser Val Val Gln Phe Pro Ser Gly Asn	144
	+ #
TTC TCC TTG ACA GCA GAA ACA GAG GAA AGG AAT TTC CCT CAA GAA AAT	480
Phe Ser Leu Thr Ala Glu Thr Glu Glu Arg Asn Phe Pro Gln Glu Asn	160
GAA CAT CTC GTG CGC TGG GCC CAA AAG GAA TAT GGA GCA GTG ACT TCG	528
Glu His Leu Val Arg Trp Ala Gln Lys Glu Tyr Gly Ala Val Thr Ser	176
TTC ACT GAA CTC AAG ATA GCA AGA AAC ATC TAT ATT AAA GTG GGA GAA	576
Phe Thr Glu Leu Lys Ile Ala Arg Asn Ile Tyr Ile Lys Val Gly Glu	192

FIGURE 1B

GAT CAA GTG TTT CCT CCT ACG TGT AAC ATA GGG AAG AAT TTC CTC TCA	624
Asp Gln Val Phe Pro Pro Thr Cys Asn Ile Gly Lys Asn Phe Leu Ser	208
&	
CTC AAT TAC CTT GCC GAG TAC CTT CAA CCC AAA GCC GCC GAA GGT TGT	672
Leu Asn Tyr Leu Ala Glu Tyr Leu Gln Pro Lys Ala Ala Glu Gly Cys	224
&	
GTC CTG CCC AGT CAG CCC CAT GAA AAG GAA GTA CAC ATC ATC GAG TTA	720
Val Leu Pro Ser Gln Pro His Glu Lys Glu Val His Ile Ile Glu Leu	240
ATT ACC CCC AGC TCG AAC CCT TAC AGC GCT TTC CAG GTG GAT ATA ATA	768
Ile Thr Pro Ser Ser Asn Pro Tyr Ser Ala Phe Gln Val Asp Ile Ile	256
GTT GAC ATA CGA CCT GCT CAA GAG GAT CCC GAG GTG GTC AAA AAC CTT	816
Val Asp Ile Arg Pro Ala Gln Glu Asp Pro Glu Val Val Lys Asn Leu	272
GTC CTG ATC TTG AAG TGC AAA AAG TCT GTC AAC TGG GTG ATC AAG TCT	864
Val Leu Ile Leu Lys Cys Lys Lys Ser Val Asn Trp Val Ile Lys Ser	288
&	
TTT GAC GTC AAG GGA AAC TTG AAA GTC ATT GCT CCC AAC AGT ATC GGC	912
Phe Asp Val Lys Gly Asn Leu Lys Val Ile Ala Pro Asn Ser Ile Gly	304
TTT GGA AAA GAG AGT GAA CGA TCC ATG ACA ATG ACC AAA TTG GTA AGA	960
Phe Gly Lys Glu Ser Glu Arg Ser Met Thr Met Thr Lys Leu Val Arg	320
GAT GAC ATC CCT TCC ACC CAA GAG AAT CTG ATG AAG TGG GCA CTG GAC	1008
Asp Asp Ile Pro Ser Thr Gln Glu Asn Leu Met Lys Trp Ala Leu Asp	336
AAT GGC TAC AGG CCA GTG ACG TCA TAC ACA ATG GCT CCC GTG GCT AAT	1056
Asn Gly Tyr Arg Pro Val Thr Ser Tyr Thr Met Ala Pro Val Ala Asn	352
AGA TTT CAT CTT CGG CTT GAG AAC AAC GAG GAG ATG AGA GAT GAG GAA	1104
Arg Phe His Leu Arg Leu Glu Asn Asn Glu Glu Met Arg Asp Glu Glu	368
GTC CAC ACC ATT CCT CCT GAG CTT CGT ATC CTG CTG GAC CCT GAC CAC	1152
Val His Thr Ile Pro Pro Glu Leu Arg <u>Ile Leu Leu Asp Pro Asp His</u>	384
peptide 1	
CCG CCC GCC CTG GAC AAC CCA CTC TTC CCA GGA GAG GGA AGC CCA AAT	1200
<u>Pro Pro Ala Leu</u> Asp Asn Pro Leu Phe Pro Gly Glu Gly Ser Pro Asn	400
GGT GGT CTC CCC TTT CCA TTC CCG GAT ATC CCC AGG AGA GGC TGG AAG	1248
Gly Gly Leu Pro Phe Pro Phe Pro Asp Ile Pro Arg Arg Gly Trp Lys	416
GAG GGC GAA GAT AGG ATC CCC CGG CCA AAG CAG CCC ATC GTT CCC AGT	1296
Glu Gly Glu Asp Arg Ile Pro Arg Pro Lys <u>Gln Pro Ile Val Pro Ser</u>	432
peptide 2	

FIGURE 1C

GTT CAA CTG CTT CCT GAC CAC CGA GAA CCA GAA GAA GTG CAA GGG GGC	1344
<u>Val Gln</u> Leu Leu Pro Asp His Arg Glu Pro Glu Glu Val Gln Gly Gly	448
GTG GAC ATC GCC CTG TCA GTC AAA TGT GAC CAT GAA AAG ATG GTC GTG	1392
Val Asp Ile Ala Leu Ser Val Lys Cys Asp His Glu Lys Met Val Val	464
&	
GCT GTA GAC AAA GAC TCT TTC CAG ACC AAT GGC TAC TCA GGG ATG GAG	1440
Ala Val Asp Lys Asp Ser Phe Gln Thr Asn Gly Tyr Ser Gly Met Glu	480
+	
CTC ACC CTG TTG GAT CCT TCG TGT AAA GCC AAA ATG AAT GGT ACT CAC	1488
Leu Thr Leu Leu Asp Pro Ser Cys Lys Ala Lys Met Asn Gly Thr His	496
&	
TTT GTT CTC GAG TCT CCC CTG AAT GGC TGT GGT ACT CGA CAT CGG AGG	1536
Phe Val Leu Glu Ser Pro Leu Asn Gly Cys Gly Thr Arg His Arg Arg	512
&	
TCG ACC CCG GAT GGT GTG GTT TAC TAT AAC TCT ATT GTG GTG CAG GCT	1584
Ser Thr Pro Asp Gly Val Val Tyr Tyr Asn Ser Ile Val Val Gln Ala	528
CCG TCC CCT GGG GAT AGC AGT GGC TGG CCT GAT GGC TAT GAA GAC TTG	1632
Pro Ser Pro Gly Asp Ser Ser Gly Trp Pro Asp Gly Tyr Glu Asp Leu	544
+++	
GAG TCA GGC GAT AAT GGA TTT CCT GGA GAC GGG GAT GAA GGA GAA ACT	1680
Glu Ser Gly Asp Asn Gly Phe Pro Gly Asp Gly Asp Glu Gly Glu Thr	560
+	
GCC CCC CTG AGC CGA GCT GGA GTG GTG GTG TTT AAC TGC AGC TTG CGG	1728
Ala Pro Leu Ser Arg Ala Gly Val Val Val Phe Asn Cys Ser Leu Arg	576
# &	
CAG CTG AGG AAT CCC AGT GGC TTC CAG GGC CAG CTC GAT GGA AAT GCT	1776
Gln Leu Arg Asn Pro Ser Gly Phe Gln Gly Gln Leu Asp Gly Asn Ala	592
#	
ACC TTC AAC ATG GAG CTG TAT AAC ACA GAC CTC TTT CTG GTG CCC TCC	1824
Thr Phe Asn Met Glu Leu Tyr Asn Thr Asp Leu Phe Leu Val Pro Ser	608
CCA GGG GTC TTC TCT GTG GCA GAG AAC GAG CAT GTT TAT GTT GAG GTG	1872
Pro Gly Val Phe Ser Val Ala Glu Asn Glu His Val Tyr Val Glu Val	624
TCT GTC ACC AAG GCT GAC CAA GAT CTG GGA TTC GCC ATC CAA ACC TGC	1920
Ser Val Thr Lys Ala Asp Gln Asp Leu Gly Phe Ala Ile Gln Thr Cys	640
&	

FIGURE 1D

TTT CTC TCT CCA TAC TCC AAC CCA GAC AGA ATG TCT GAT TAC ACC ATC	1968
Phe Leu Ser Pro Tyr Ser Asn Pro Asp Arg Met Ser Asp Tyr Thr Ile	656
ATC GAG AAC ATC TGT CCG AAA GAC GAC TCT GTG AAG TTC TAC AGC TCC	2016
Ile Glu Asn Ile Cys Pro Lys Asp Asp Ser Val Lys Phe Tyr Ser Ser	672
&	
AAG AGA GTG CAC TTT CCC ATC CCG CAT GCT GAG GTG GAC AAG AAG CGC	2064
Lys Arg Val His Phe Pro Ile Pro His Ala Glu Val Asp Lys Lys Arg	688
TTC AGC TTC CTG TTC AAG TCT GTG TTC AAC ACC TCC CTG CTC TTC CTG	2112
Phe Ser Phe Leu Phe Lys Ser Val Phe Asn Thr Ser Leu Leu Phe Leu	704
#	
CAC TGC GAG TTG ACT CTG TGC TCC AGG AAG AAG GGC TCC CTG AAG CTG	2160
His Cys Glu Leu Thr Leu Cys Ser Arg Lys Lys Gly Ser Leu Lys Leu	720
&	
CCG AGG TGT GTG ACT CCT GAC GAC GCC TGC ACT TCT CTC GAT GCC ACC	2208
Pro Arg Cys Val Thr Pro Asp Asp Ala Cys Thr Ser Leu Asp Ala Thr	736
&	
ATG ATC TGG ACC ATG ATG CAG AAT AAG AAG ACA TTC ACC AAG CCC CTG	2256
Met Ile Trp Thr Met Met Gln Asn Lys Lys Thr Phe Thr Lys Pro Leu	752
GCT GTG GTC CTC CAG GTA GAC TAT AAA GAA AAT GTT CCC AGC ACT AAG	2304
Ala Val Val Leu Gln Val Asp Tyr Lys Glu Asn Val Pro Ser Thr Lys	768
GAT TCC AGT CCA ATT CCT CCT CCT CCT CCA CAG ATT TTC CAT GGC CTG	2352
Asp Ser Ser Pro Ile Pro Pro Pro Pro Pro Gln Ile Phe His Gly Leu	784
GAC ACG CTC ACC GTG ATG GGC ATT GCA TTT GCA GCA TTT GTG ATC GGA	2400
Asp Thr Leu Thr Val Met Gly Ile Ala Phe Ala Ala Phe Val Ile Gly	800
GCG CTC CTG ACG GGG GCC TTG TGG TAC ATC TAC TCC CAC ACA GGG GAG	2448
Ala Leu Leu Thr Gly Ala Leu Trp Tyr Ile Tyr Ser His Thr Gly Glu	816
ACA GCA CGA AGG CAG CAA GTC CCT ACC TCG CCG CCA GCC TCG GAG AAC	2496
Thr Ala Arg Arg Gln Gln Val Pro Thr Ser Pro Pro Ala Ser Glu Asn	832
\$ @	
AGC AGC GCG GCC CAC AGC ATC GGC AGC ACT CAG AGT ACC CCC TGC TCT	2544
Ser Ser Ala Ala His Ser Ile Gly Ser Thr Gln Ser Thr Pro Cys Ser	848
AGC AGC AGC ACA GCC TAGGTGGACA GACAGACGCC CGCCCACCGC AGCCAGGGCA	2599
Ser Ser Ser Thr Ala***	853

FIGURE 1E

GGGCCCAGTG CCAGTGCTGC GTGTCCACAG TCAGAAGTCT TGATCTGGGC TCCCTGTAAA 2659  
GAAAGAGTGA ATTTAGTAT ACAGACAGCC AGTTCTACCC ACCCCTTACC ACGGCCCACA 2719  
TAAATGTGAC CCTGGGCATC TGTCACACGA AAGCTAAGCT GGTGGCCTTC CCCACCAGCC 2779  
CCTCGCAGGA TGGGGGTTTC AATGTGAAAC ATCTGCCAGT TTTGTTTTGT TTTTTTAATG 2839  
CTGCTTTGTC CAGGTGTCCA AACATCCATC ATTTGGGGTG GTCTGTTTTA CAGAGTAAAG 2899  
GAGGCGGTGA AGGGACGTCA GCTAGTGTGT AGAGCCAAGG GGAGACAGCT AGGATTCTCG 2959  
CCTAGCTGAA CCAAGGTGTA AAATAGAAGA CACGCTCC 2997

FIGURE 2

AGTTTCCTGT	TTCCCCCGCA	GCGCTGAGTT	GAAGTTGAGT	GTTGGCGAGG
CGCGCACGGA	GCGACGACAC	CCCCGCGGT	GCACCCGCTC	GAGTCACTCG
CCGGACTCCT	GTGCAGCTTC	CCTCGGCCGC	CGGGGGCCTC	GGGACAGGAG
GCCGGCCTCC	AGGCCCTCC	TGGCTGGCGA	GCGGGCGCCA	CCCGCGCCTC
GCACATCTGC	GCTGCCGGCC	CGGCGCGGGG	TCCGGAGAGG	CATCTGGCCC
GAGCGCAGCC	AGGGGTCCGG	GAAGGCGCCG	TCCGTGCGCT	GCGCGGCGCG
TCTATGACGA	GCAGCGGGGT	CTGCCATGGG	TCGGGGGCTG	GGGGGCTCGG
TGTGGCCGCT	GCACATCGTC	CTGTGGACGC	GTATCGCCAG	CTCAGGGGCC
CCGCACGTTT	AGAAGTCGGT	TAATAACGAC	ATGATAGTCA	CACGATCCCA
CGGTGCAGTC	AAGTTTCCAC	AACTGTGTAA	ATTTTGTGAT	CTGACAACAA
CCACCTGTGA	CAACCAGAAA	TCCTGCATGA	GCAACTGCAG	GTGAGATTTT
ATCTGTGAGA	AGCCACAGGA	AGTCTGTGTG	GCTGTATGGA	CATCACCTCC
CGAGAACATA	ACACTAGAGA	CAGTTTGCCA	TGACCCCAAG	GAAAGAATGA
ATGACTTTAT	TCTGGAAGAT	GCTGCTTCTC	CAAAGTGCAT	CTCCCCTACC
AAAAAAAAGC	CTGGTGAGAC	TTTCTTCATG	TGTTCTGTGA	TATGAAGGAA
GTGCAATGAC	AACATCATCT	TCTCAGAAGA	ATATAACACC	GCTCTGATGA
ACTTGTGCT	AGTCATATTT	CAAGTGACAG	GCATCAGCCT	AGCAATCCTG
CTGGGAGTTG	CCATATCTGT	CATCATCATC	TTCTACTGCT	CCTGCCACCA
CCGGCAGCAG	AAGCTGAGTT	CAACCTGGGA	AACCGGCAAG	ACCGCGTTAA
TCATGGAGTT	CAGCGAGCAC	TGTGCCATCA	TCCTGGAAGA	ACGCGGAAGC
GACATCAGCT	CCACGTGTGC	CAACAACATC	AACCACAACA	TGACCGCTCT
GCCCATTGAG	CTGGACACCC	TGGTGGGGAA	AGGTGCTTTT	CAGAGTGCCT
ATAAGGCCAA	GCTGAAGCAG	AACACTTCAG	AGCAGTTTGA	GCTGAGGTCT
GTCAAGATCT	TTCCCTATGA	GGAGTATGCC	TCTTGGAAGA	GACAGTGGCA
CATCTTCTCA	GACATCAATC	TGAAGCATGA	GAACATACTC	CAGAGAAGGA
CGGCTGAGGA	GCGGAAGACG	GAGTTGGGGA	AACAATACTG	CAGTTCTCTGA
GCCTTCCACG	CCAAGGGCAA	CCTACAGGAG	TACCTGACGC	GCTGATCACC
CAGCTGGGAG	GACCTGCGCA	AGCTGGGCAG	CTCCCTCGCC	GGCATGTCAT
CTCACCTCCA	CAGTGATCAC	ACTCCATGTG	GGAGGCCCAA	CGGGGGATTG
GTGCACAGGG	ACCTCAAGAG	CTCCAATATC	CTCGTGAAGA	GATGCCCATC
CTGCTGCCTG	TGTGACTTTG	GGCTTTCCCT	GCGTCTGGAC	ACGACCTAAC
CTGTGGATGA	CCTGGCTAAC	AGTGGGCAGG	TGGGAACCTGC	CCTACTCTGT
GCTCCAGAAG	TCCTAGAATC	CAGGATGAAT	TTGGAGAATG	AAGATACATG
CAAGCAGACC	GATGTCTACT	CCATGGCTCT	GGTGCTCTGG	CTGAGTCCTT
CTCGCTGTAA	TGCAGTGGGA	GAAGTAAAG	ATTATGAGCC	GAAATGACAT
TCCAAGGTGC	GGGAGCACCC	CTGTGTGCAA	AGCATGAAGG	TCCATTTGGT
GAGAGATCGA	GGGCGACCAG	AAATTCCAG	CTTCTGGCTC	ACAACGTGTT
GCATCCAGAT	GGTGTGTGAG	ACGTTGACTG	AGTGCTGGGA	AACCACCAGG
GAGGCCCGTC	TCACAGCCCA	GTGTGTGGCA	GAACGCTTCA	CCACGACCCA
GCATCTGGAC	AGGCTCTCGG	GGAGGAGCTG	CTCGGAGGAG	GTGAGCTGGA
AAGACGGCTC	CCTAAACACT	ACCAAATAGC	TCTTATGGGG	AAGATTCCCTG
ATGTCCAAAG	AGGCTGCCCC	TCTCACCAAA		CAGGCTGGGC

7/7

FIGURE 3

MGRGLLRGLW	PLHIVLWTRI	ASTIPPHVQK	SVNNDMIVTD	NNGAVKFPQL
CKFCDVRFST	CDNQKSCMSN	CSITSICEKP	QEVCAVAVRK	NDENITLETV
CHDPKLPYHD	FILEDAASPK	CIMKEKKKPG	ETFFMCSCSS	DECNDNIIFS
EEYNTSNPDL	LLVIFQVTGI	SLLPPLGVAI	SVIIIFYCYR	VNRQOKLSST
WETGKTRKLM	EFSEHCAIIL	EDDRSDISST	CANNINHNTTE	LLPIELDTLV
GKGRFAEVYK	AKLKQNTSEQ	FETVAVKIFP	YEEYASWKTE	KDIFSDINLK
HENILQFLTA	EERKTELGKQ	YWLITAFHAK	GNLQEYLTRH	VISWEDLRKL
GSSLARGIAH	LHSDHTPCGR	PKMPIVHRDL	KSSNILVKND	LTCCLCDFGL
SLRLDPTLSV	DDLANSQVVG	TARYMAPEVL	ESRMNLENAE	SFKQTDVYSM
ALVLWEMTSR	CNAVGEVKDY	EPPFGSKVRE	HPCVESMKDN	VLRDRGRPEI
PSFWLNHQGI	QMV CETLTEC	WDHDPEARLT	AQCVAERFSE	LEHLDRLSGR
SCSEEKIPED	GSLNTTK			

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C12N15/12; C12N5/10; C12P21/08; C07K13/00 A61K37/02		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C07K ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP,A,0 369 861 (ONCOGEN LTD PARTNERSHIP, US) 23 May 1990 see the whole document ---	1-41
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS. vol. 179, no. 1, 30 August 1991, DULUTH, MINNESOTA US pages 378 - 385 O'GRADY P; HUANG SS; HUANG JS; 'Expression of a new type high molecular weight receptor (type V receptor) of transforming growth factor beta in normal and transformed cells.' see the whole document --- <div style="text-align: right;">-/--</div>	1,16,29
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
20 JANUARY 1993		12.02.93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		S.A. NAUCHE



## III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	<p>CELL. vol. 68, no. 4, 21 February 1992, CAMBRIDGE, MA US pages 775 - 785 LIN HY;WANG XF;NG-EATON E;WEINBERG RA;LODISH HF; 'Expression cloning of the TGF-beta type II receptor, a functional transmembrane serine/threonine kinase.' see the whole document</p> <p>---</p>	<p>1-3,6,7, 10,11, 14-16, 19,20, 24-26, 28-31</p> <p>37-41</p>
P,X	<p>CELL. vol. 67, no. 4, 15 November 1991, CAMBRIDGE, MA US pages 785 - 795 LOPEZ-CASILLAS F;CHEIFETZ S;DOODY J;ANDRES JL;LANE WS;MASSAGUE J; 'Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system.' see the whole document</p> <p>---</p>	<p>1-5,8,9, 12,13, 16-18, 21-23, 25-36,41</p>
P,X	<p>CELL. vol. 67, no. 4, 15 November 1991, CAMBRIDGE, MA US pages 797 - 805 WANG XF;LIN HY;NG-EATON E;DOWNWARD J;LODISH HF;WEINBERG RA; 'Expression cloning and characterization of the TGF-beta type III receptor.' see the whole document</p> <p>---</p>	<p>1-5,8,9, 12,13, 16-18, 21-23, 25-36,41</p>
A	<p>JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 265, no. 33, 25 November 1990, BALTIMORE, MD US pages 20533 - 20538 CHEIFETZ S;HERNANDEZ H;LAIHO M;TEN DIJKE P;IWATA KK;MASSAGUE J; 'Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms.'</p> <p>---</p>	
A	<p>JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 263, no. 32, 15 November 1988, BALTIMORE, MD US pages 16984 - 16991 CHEIFETZ S.; ANDRES J.L.; MASSAGUE J; 'The transforming growth factor-beta receptor type III is a membrane proteoglycan. Domain structure of the receptor.'</p> <p>---</p>	

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A!	<p>ANNALS OF THE NEW YORK ACADEMY OF SCIENCES vol. 593, 1990, NEW YORK, US; pages 59 - 72 MASSAGUÉ, J. ET AL; 'TGF-Beta receptors and TGF-beta binding proteoglycans : recent progress in identifying their functional properties.'</p> <p>-----</p>	

US 9209326  
SA 66669

**20/01/93**

[illegible]

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**